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Original Research Article

Effect of aqueous seed extract of fenugreek (*Trigonella foenum-graecum* L.) on milk production in rats

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Abstract

Purpose: *To evaluate the effect of fenugreek (Trigonella foenum-graecum L., Fabaceae) aqueous seed extract on milk production in rats and to assess its phytoconstituents.*
Method: *Three groups of six lactating rats were used. Each lactating rat and her six pups were kept apart. From lactation day 3 to 17, each group received a daily single oral dose of distilled water, metoclopramide (5 mg/kg), or aqueous seed extract of fenugreek (200 mg/kg). Three groups of six virgin rats also received the same treatment for 14 days. An indirect technique of daily milk produced for weight gain of pups was used to assess milk production. Serum prolactin levels in virgin rats were determined on days 1, 7 and 14. Bioactive compounds of fenugreek seed extract were identified by ultra-performance liquid chromatography.*
Results: *On day 14, fenugreek seed extract and metoclopramide significantly increased prolactin levels in virgin rats ($p < 0.01$, $p < 0.001$), but only metoclopramide significantly increased milk production and pup weight gain ($p < 0.05$). Chromatographic analysis revealed diverse phytoconstituents of flavonoids, carbohydrates and amino acids.*
Conclusion: *Fenugreek seed extract significantly influences serum prolactin levels in virgin rats but does not affect the milk production of lactating rats.*

Keywords: *Galactagogue, Fenugreek, Metoclopramide, Milk production, Prolactin*

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INTRODUCTION

Milk is secreted from mammary glands of healthy postpartum females in response to suckling in a process called lactation. Breast milk is the only known ideal food for infants and a baby requires the mother's milk for first six months [1]. World Health Organization recommends that mothers completely breastfeed their infants for six months and extend it to more than two years with proper complementary solid food. According to a United Nations Children's Fund report in 2020, 26 % of children were exclusively breastfed during the first six months of life. An issue that has dominated lactation field for many years is hypogalactia or reduced milk production. It is the most frequent cause of breastfeeding failure, leading to cessation of breastfeeding [2]. According to reports, > 20 % of mothers who breastfed their infants experienced postpartum hypogalactia. Incidence of hypogalactia among women has increased because of rising average age of mothers and growing number of cesarean sections globally [3].

Complementary traditional medicines are used in many nations. Nowadays, the use of herbal galactagogues is increasing as an alternative to drugs in order to enhance milk production. There are several reports on relation between herbal medicine and breastfeeding. These reports usually focus on effects rather than mechanisms by which milk production is stimulated. More than one lactogenic study has been reported for fennel (*Foeniculum vulgare* M.), ginger (*Zingiber officinale* R.), asparagus (*Asparagus racemosus* W.), anise (*Pimpinella anisum* L.), barley (*Hordeum vulgare* L.), moringa (*Moringa oleifera* L.), galega (*Galega officinalis* L.), marshmallow (*Althaea officinalis* L.), nettle (*Urtica dioica* L.) and others [4]. Fenugreek (*Trigonella foenum-graecum* L.) is a potent medicinal plant. The seed and aerial parts of this small legume belonging to the family Fabaceae have been used for centuries as a protein source in human and animal nutrition and traditional medicine. Therefore, it is well known for its broad nutritional and medicinal effects [5]. In addition to its medicinal properties, the spice is valued for its culinary applications as it enhances the flavor of food [6]. In Jordanian folk medicine, this therapeutic herb is commonly used for its diuretic, cardiotonic, hypotensive, hypoglycemic and hypolipidemic effects [7].

Galactagogues are well-documented for their anecdotal benefits in enhancing milk supply and they are often used instead of pharmaceutical medications in the Middle East. Among these, fenugreek has become the most widely utilized natural galactagogue in the region, even surpassing its use in Jordan [8]. Hence, the present study aimed to assess the effect of aqueous seed extract of fenugreek on milk production in rats and to identify its phytoconstituents.

EXPERIMENTAL

Plant materials

Fenugreek seeds were purchased from local traditional herbal market in Zarqa, Jordan, in 2022. The plant was taxonomically identified by direct comparison with authenticated samples with the assistance of Associate Professor Mohammad Al-Gharaibeh, Faculty of Agriculture, Jordan Department of Plant Production, University of Science and Technology. The specimen (with voucher no. Fenk-2022) was deposited at the Pharmacognosy and Phytochemistry Laboratory, Faculty of Pharmacy, Zarqa University.

Preparation of fenugreek seed extract

To mimic as closely as possible the methods commonly used by laymen, fenugreek aqueous seed extract was prepared by decoction and maceration. Briefly, 300 g of finely ground fenugreek seeds were boiled in 300 mL of distilled water for 15 min, mixed and left to stand for 24 h with continuous shaking [9]. The solution was filtered and lyophilized, and the extract was stored in a sterile container at - 4 °C until required. The extraction yield was 3.7 % of fenugreek.

Animals

Female Wistar laboratory rats (weight 170 – 200 g) were housed in cages at 22 – 25 °C and 50 – 60 % relative humidity (controlled conditions). They were allowed access to standard pellet food and water *ad libitum.* All procedures involving animal care and use were conducted in accordance with standard ethical guidelines and all protocols were approved by the Research and Ethics Committee, Faculty of Pharmacy, Applied Science University, Amman, Jordan (approval no. 2022-PHA-23).

Effect of fenugreek seed extract on milk production

Eighteen female Wistar rats were mated with male rats several days before parturition in controlled conditions. Each pregnant female Wistar rat was separated from the others. Rats were permitted to deliver their young. Immediately after parturition, mothers were separated from their pups. The number of pups per lactating rat was six and the day of parturition was day 1 of lactation [10].

Design

The design followed a previous study that divided lactating rats into three groups [10]. Each group comprised six lactating rats. Lactating rats were treated from day 3 to day 17 as follows: Group I received water *ad libitum* and served as a negative control, group II was treated with standard drug, metoclopramide (5 mg/kg), and Group III was treated with fenugreek seed extract (200 mg/kg). Distilled water was used as a solvent for metoclopramide and fenugreek seed extract. The prepared solution was administered to experimental rats by oral gavage. Dose of metoclopramide (5 mg/kg) was selected based on previous studies [11]. Although pups were not treated, their weights were determined daily at different times of the day from day 3 to day 17.

Determination of daily milk production and body weight

After spending the previous night with their mothers, pup weight (P1) was determined daily at 8:00 AM. Pups were immediately separated from lactating rats for 4 h. They were weighed again (P2) at 12:00 PM and returned to nursing rats for 1 h of lactation. At 1:00 PM daily, the third weight (P3) was taken to determine the amount of milk pups had consumed. The weight difference in pups before and after feeding sessions was used to calculate milk yield. This procedure was performed daily from day 3 to day 17 of lactation. Milk yield was computed using Eq 1 [12].

Milk yield (g) = (P3 − P2) + ((P2 − P1)/4)…….. (1)

where (P3 − P2) is weight gain of pups after lactation and (P2 − P1)/4 is weight loss correlation coefficient.

Evaluation of serum prolactin

For 14 days, 18 virgin rats weighing 170 – 200 g were divided into three groups of six animals each. Grouping (I, II and III) and treatments of virgin rats were the same as those of lactating rats. Fenugreek seed extract and metoclopramide were orally administered to virgin rats daily in a single dose for 14 days. The serum prolactin levels were determined on days 1, 7 and 14. Blood samples were collected from the retro-orbital plexus of rats and centrifuged at 1000 rpm for 20 min [12]. The serum prolactin level was assayed using immunoenzymatic method (ELISA kit) after the supernatant was collected [13].

Identification of bioactive compounds in fenugreek seed extract

Bioactive compound identification was performed by ultra-performance liquid chromatography (UPLC) quadrupole time-of-flight mass spectroscopy (Q-TOF/MS). The chromatographic conditions are summarized in Table 1. Apollo II ion funnel electrospray source was used as a source in both positive and negative modes. Mass spectrometry conditions were as follows: capillary voltage was 2500 V, nebulizer gas was at 2.0 bar, dry gas (nitrogen) flow was 8 L/min, and dry temperature was 200 °C. Mass accuracy was < 1 ppm and mass resolution was 50000 full sensitivity resolution (FSR). Time of flight (TOF) repetition rate was up to 20 kHz and MS data were analyzed by Data Analysis 4.2 (Bruker Daltonics, Bremen, Germany).

Statistical analysis

For all tested parameters, a one-way analysis of variance followed by Dunnett's *post-hoc* test was performed using IBM SPSS v.22.0. The data are presented as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant, $p < 0.01$ was considered statistically very significant and $p < 0.001$ was considered statistically highly significant.

RESULTS

Effect of fenugreek seed extract on milk production

Milk produced daily by lactating rats was measured indirectly via pup weight. Data showed that milk yield of lactating rats in each experimental group was not proportional to number of days of lactation (Figure 1 A).

Table 1: Summary of chromatographic conditions

UPLC condition	Injection volume	Autosampler temperature	Column temperature	Total run time
	3 µL	***8 °C***	***40 °C***	***35 min***
Chromatography	Mobile phase	Solvents: (A) Water with 0.05% formic acid (B) Acetonitrile Gradient: 0–27 min linear gradient from 5–80% B; 27–29 min 95% B; 29.1 min 5% B		
	Column type	Bruker Daltonics C-18 column (100 × 2.1 mm × 1.8 µm; 120 Aº)		

Compared with the negative control, fenugreek seed extract did not significantly increase milk production ($p > 0.05$). However, metoclopramide significantly increased milk yield in lactating rats ($p < 0.05$; Figure 1 B).

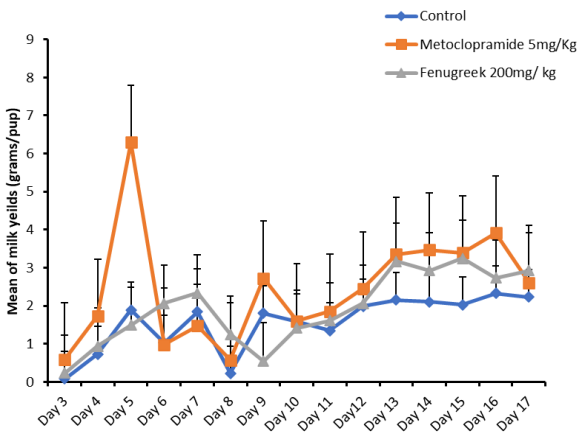

Figure 1A: Effect of fenugreek seed extract on daily milk production in female Wistar rats from day 3 to day 17 of lactation

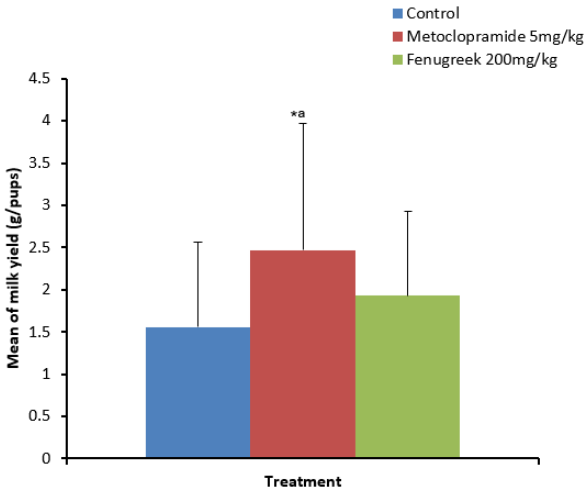

Figure 1B: Effect of fenugreek seed extract on average milk quantity in lactating Wistar rats from day 3 to day 17 of lactation. *P < 0.05, a: vs control

Effect of fenugreek seed extract on pup weight gain

In comparison with control group, metoclopramide significantly increased the weight of pups (p < 0.05), whereas fenugreek seed extract did not significantly increase the weight of pups (p > 0.05; Figure 2).

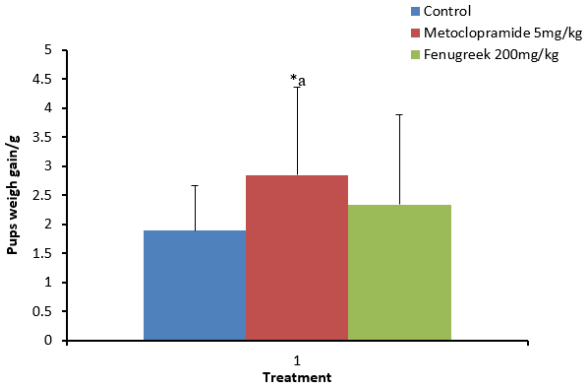

Figure 2: Effect of fenugreek seed extract on pup average weight gain from day 3 to day 17 of lactation. *P < 0.05, a: vs control

Serum prolactin levels

Table 2 shows the effect of fenugreek seed extract on serum prolactin levels in virgin female rats. At day 1, prolactin levels in all groups were less than 0.9 ng/mL. However, fenugreek seed extract and metoclopramide groups had significantly higher prolactin levels on days 7 and 14 compared with control group (p < 0.01 and p < 0.001, respectively).

Bioactive compounds in fenugreek seed extract

Several bioactive compounds were identified in fenugreek seed extract. The complete list of identified compounds along with their phytochemical class and chemical formula are presented in Table 3.

DISCUSSION

Mother's milk is crucial for newborn nutrition, directly affecting neonatal growth, development and health. Metoclopramide is a commonly used galactagogue in humans but it has serious side effects in nursing mothers, including gastrointestinal disorders, insomnia, severe depression and seizures [14].

Table 2: Effect of fenugreek seed extract on serum prolactin levels in virgin female rats

Study period	Control	Metoclopramide (5 mg/kg)	Fenugreek seed extract (200 mg/kg)
Day 1	<0.9	<0.9	<0.9
Day 7	<0.9	0.98±0.35[a]	1.27±0.28[a]
Day 14	<0.9	6.9±1.40***[a]	3.1±1.55**[a]

***P < 0.01, ***P < 0.001. a: vs control*

Table 3: Phytoconstituents and their phytochemical class identified in fenugreek seed extract

S/no	RT	Identified compound name	Phytochemical class	Chemical formula	Ion mode
1.	0.53	L-Lysine	Amino acid	$C_6H_{14}N_2O_2$	(MH)+
2.	0.60	Sucrose	Carbohydrate	$C_{12}H_{22}O_{11}$	(MH)+
3.	0.60	Raffinose	Carbohydrate (Trisaccharide)	$C_{18}H_{32}O_{16}$	(MH)+
4.	0.60	Glutamic acid	Amino acid	$C_5H_9NO_4$	(MH)+
5	0.60	L-Alanine	Amino acid	$C_3H_7NO_2$	(MH)+
6.	1.00	Succinic acid	Carboxylic acid	$C_4H_6O_4$	(M-H)-
7.	1.02	L-Leucine	Amino acid	$C_6H_{13}NO_2$	(MH)+
8.	1.02	Valine	Amino acid	$C_5H_{11}NO_2$	(M-H)-
9.	1.34	Phenylalanine	Amino acid	$C_9H_{11}NO_2$	(M-H)-
10.	2.70	L-Tryptophan	Amino acid	$C_{11}H_{12}N_2O_2$	(MH)+
11.	4.10	Riboflavine	Vitamin	$C_{17}H_{20}N_4O_6$	(MH)+
12.	4.07	Umbelliferone	Coumarin	$C_9H_6O_3$	(MH)+
13.	4.66	Saponarin	Flavone glycoside	$C_{27}H_{30}O_{15}$	(M-H)-
14.	4.88	Apiin	Flavonoid	$C_{26}H_{28}O_{14}$	(MH)+
15.	5.70	Vitexin	Flavonoid	$C_{21}H_{20}O_{10}$	(MH)+
16.	9.90	9,12-Octadecadienoic acid (conjugated linoleic acid)	Polyunsaturated fatty acid	$C_{18}H_{32}O_2$	(M-H)-

Compounds are listed in order of retention time (RT, min)

The use of herbal remedies has significantly increased milk production in humans, cows and goats; thus, is considered vital for human breastfeeding medicine and animal dairy production [2]. Traditional medicine has examined and used medicinal plants that are dispersed in the Mediterranean region. Fenugreek has long been used as a culinary ingredient and a medicinal component. It has also been used as a galactagogue and in traditional medicine to treat a variety of diseases [15]. The focus of this study was on phytotherapy — use of fenugreek in traditional medicine as a complementary form of primary healthcare. However, more pharmacological investigations on efficacy of these plants are required to supplement traditional knowledge.

Milk produced by rats is difficult to determine. Thus, weighing pups at various intervals is used to indirectly ascertain the volume of milk produced [12]. In this study, rat milk production was determined by weighing pups daily and the routine was discontinued on day 17 when pups started eating solid food. The milk yield of rats in each treatment group was not proportional to the number of days of lactation. This finding is consistent with other studies that evaluated galactagogue potential of *Musa paradisiaca* (Musaceae), *Euphorbia hirta* (Euphorbiaceae), *Pimpinella anisum* (Umbelliferae) and *Nigella sativa* (Ranunculaceae) [16,17]. The non-linearity of milk production versus days of lactation may be explained by the fact that mammary glands undergo an alternation of proliferation and apoptosis of mammary cells during lactation. Hence, there is a variation in the kinetics of milk biosynthesis depending on the lactation phase [18].

In addition, following daily treatment of virgin rats, serum prolactin assays were performed on days 1, 7 and 14 to determine the mechanism of galactagogue activity. Prolactin is known to cause an increase in expression of surface receptors of alveolar cells. This increased expression encourages passage of nutrients and water into alveolar cells to produce milk [18]. Virgin rats, rather than lactating rats, were used because lactating mothers already exhibit elevated prolactin levels, which could introduce bias into results. Prolactin secretion was stimulated in the treated groups of virgin rats on days 7 and 14, and fenugreek seed extract significantly induced serum prolactin levels. These results reflect those of Cox *et al* [19], who also found a non-significant relationship between the concentration of plasma prolactin and the short-term rate of milk synthesis. Results of this study showed that rats given fenugreek seed extract produced milk, but not significantly more than the negative control group. Weight gain by pups is an indicator of milk production in female rats [11]. In the present study, fenugreek seed extract did not significantly increase the weight gain of pups ($p > 0.05$). Thus, there was a link between not gaining weight and insufficient milk production.

Furthermore, two major changes occur during lactation. First, the formation of a physiological control of sucking behavior after day 10 postpartum. Second, a changing receptivity to a nipple or milk ejection characteristic of the mother after day 14 postpartum [20,21]. Animal

studies suggest that fenugreek primarily works by raising insulin and oxytocin secretion. However, the galactagogue effect of fenugreek may be primarily psychological in humans [21,22]. Most evidence for the galactagogue effect is anecdotal. A few studies have produced conflicting findings regarding the galactagogue impact of fenugreek [22]. A meta-analysis of controlled studies reported that the safety profile of fenugreek is not clear and that it has a low galactagogue effect [23]. Some data also report that fenugreek may be more useful in the first few days following delivery rather than two weeks later [24]. However, because researchers used multi-ingredient combination products in which fenugreek was one of many components, the outcomes of some of these trials may differ from those of studies in which fenugreek was used alone [25].

Moreover, sapogenins of fenugreek (e.g., diosgenin and yamogenin) have estrogenic properties, but their exact mechanism of action is unknown. Researchers believe that breast glands are improved sweat glands and that fenugreek increases milk production by increasing sweat production. Also, fenugreek seed, which contains diosgenin, has phytoestrogenic properties. The chemical structure of phytoestrogen is similar to that of estrogen which attaches to receptors and may have estrogenic properties [26]. In the chromatographic analysis, phytoestrogens were not determined in fenugreek aqueous seed extract because they are insoluble in water. However, fenugreek seed extract showed the presence of amino acids. Administration of amino acids (e.g., tyrosine and phenylalanine) boosts prolactin production in healthy individuals. This stimulation appears to occur within the brain, possibly by preventing the synthesis or release of dopamine from the hypothalamus [27]. Therefore, this might be interpreted as a significant effect of fenugreek on prolactin levels in the current study.

Limitations of this study

The small number of rats in each group and use of only a dose of 200 mg/kg fenugreek seed extract were the major limitations of this study. Utilizing several doses and a larger sample size would likely yield more robust results.

CONCLUSION

Aqueous seed extract of fenugreek has notable effect on serum prolactin levels in virgin rats. This effect may be attributed to the bioactive compounds in seed extract. Thus, this study supports the role of medicinal plants in developing new drugs. Further studies are needed to explain the conflicting results concerning milk production levels not correlating with the serum prolactin level.

DECLARATIONS

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Ethical approval

All protocols were approved by the Research and Ethics Committee, Faculty of Pharmacy, Applied Science University, Amman, Jordan (approval no. 2022-PHA-23).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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